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Towards a Theory of Functional Magnetic Resonance Spectroscopy (fMRS): A Meta-analysis and discussion of using MRS to measure changes in neurotransmitters in real time.

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Abstract

Proton magnetic resonance spectroscopy is a powerful tool to investigate neurochemistry and physiology in vivo. Recently researchers have started to use MRS to measure neurotransmitter changes related to neural activity, so called functional MRS (fMRS). Particular interest has been placed on measuring glutamate changes associated with neural function, but differences are reported in the size of changes seen. This review discusses fMRS, and includes meta-analyses of the relative size of glutamate changes seen in fMRS, and the impact experimental design and stimulus paradigm may have. On average glutamate was found to increase by 6.97 % (±1.739%) in response to neural activation. However, factors of experimental design may have a large impact on the size of these changes. For example an increase of 4.749% (±1.45 %) is seen in block studies compared to an increase of 13.429% (± 3.59) in studies using event related paradigms. The stimulus being investigated also seems to play a role with prolonged visual stimuli showing a small mean increase in Glutamate of 2.318 %

(± 1.227%) while at the other extreme, pain stimuli show a mean stimulation effect of 14.458 % (± 3.736%). These differences are discussed with regards to possible physiologic interpretations, as well experimental design implications.

Introduction

Proton magnetic resonance spectroscopy (1H-MRS) is a powerful research and clinical tool used to investigate neurochemistry and physiology in vivo. 1H-MRS currently has clinical applications in cancer and neurometabolic disease and it is used extensively to study brain chemistry in clinical research in schizophrenia (Bustillo et al., 2010; Mullins et al., 2003; Poels et al., 2014), depression (Horn et al., 2010; Merkl et al., 2011; Price et al., 2009), Alzheimer's disease (Kantarci, 2007; 2013), traumatic brain injury (Mullins & Vink, 1995; Poole et al., 2014; Shutter, Tong, & Holshouser, 2004), addiction (Frye et al., 2016; Martinez et al., 2014; Thoma et al., 2011; Yücel et al., 2007), pain (Grachev, Fredrickson, & Apkarian, 2000; Gussew et al., 2010; Mullins, Rowland, Jung, & Sibbitt, 2005), stroke and epilepsy (Helms, 2006; Simister, McLean, Barker, & Duncan, 2003), amongst many other conditions. As a clinical research tool, standard 1H-MRS acquisition is often thought of as a "static" snapshot of neurochemistry, and as such it has often

been used as a comparative method to examine underlying differences in resting neurochemistry between clinical cohorts and healthy controls.

More recently, it has been shown that 1H-MRS may be used to investigate dynamic changes in neuronal metabolites, particularly in neurotransmitter systems (Lin, Stephenson, Xin, Napolitano, & Morris, 2012; Mangia et al., 2007; Mullins et al., 2005). There is currently a small but growing number of separate research groups working to develop this new approach, termed functional magnetic resonance spectroscopy (fMRS). The power of fMRS stems from its potential to probe neurochemical function in a dynamic way, complementing established techniques, such as functional magnetic resonance imaging (fMRI) and electroencephalography (EEG), thereby significantly broadening the scope of tractable research questions. At the same time, it enables the nuanced study of neurochemical function in vivo, with a sensitivity and in a timescale (ms after stimulus onset (Apšvalka, Gadie, Clemence, & Mullins, 2015; Lally et al., 2014)) relevant to human psychological function and behaviour. As such fMRS has the

potential to carve new research avenues, and has importance in basic and translational research as well as clinical practice.

The concept behind fMRS, the sequential acquisition of MRS spectra over time to measure changes in neurochemical concentrations, is not new. Indeed there is a large body of literature detailing the use of ^{13}C MRS in this way to follow the rate of incorporation of ^{13}C from labelled substrates into metabolically active chemicals to study metabolism and energy turn over (for review see (Rothman, De Feyter, Graaf, Mason, & Behar, 2011)), however, there is a much smaller body of work looking at the use of ^{1}H-MRS to measure neuro-metabolite dynamics. Still, even here, work has been happening for some time. One of the earliest studies from 1991 (Prichard et al., 1991) demonstrated the ability of 1H-MRS to measure changes in lactate in response to neural activity, which was shown to increase in response to prolonged visual stimulation. Since this initial report, the finding of lactate increases in response to increased neural activity has been largely confirmed (Dager et al., 1999; R. J. Maddock et al., 2006; Richards et al., 2000; 1997) with the latest 1H-MRS studies almost always reporting lactate

increases in response to prolonged neural activity (Bednařík et al., 2015; 2017; Ip et al., 2017; Lin et al., 2012; Mangia et al., 2008). However, while informative, investigating lactate changes is not the only way fMRS can be used.

In 2005 ^{1}H-MRS was extended to measure changes in neurotransmitters in response to neural activity – specifically Glutamate and Glutamine elevations in response to acute pain (Mullins et al., 2005). While this finding was at first received with some scepticism, other reports of glutamate increases during neural activity soon followed (Gussew et al., 2010; Mangia et al., 2007; Schaller, Mekle, Xin, Kunz, & Gruetter, 2013), although these varied in the effect size between an increase of 2-3% for visual stimulation to up to 18% for a painful stimulus. Taken together these studies support the existence of MRS visible glutamate increases during neural activity, and have led to the development of fMRS as a tool to study these and other neurotransmitter dynamics in real time.

This review will discuss some of the differing aspects of using 1H-MRS to measure neurotransmitter dynamics, focussing on glutamate, some of the ways fMRS has been applied to date, including some discussion of the different

behavioural and stimulation paradigms that have been employed. The review will also use meta-analytical techniques to try and gain an appreciation of the size of changes in Glutamate that might be expected in an fMRS experiment, and investigate the impact experimental factors may have on these changes. These changes will be discussed with regards to possible interpretations and experimental design implications. While this review focuses on the use of fMRS to measure Glutamate changes in response to neural activity, it is recognised that changes in other neurotransmitters and neurotransmitter precursors can, and have been studied as well.

Advantages and disadvantages of using proton MRS to investigate neurotransmitter dynamics associated with neural activity.

Using ^{1}H-MRS to measure changes in neurotransmitters in accompanying neural activity has a few advantages over other functional imaging approaches. The first is that it can be done in a totally non-invasive manner and does not require the injection of labeled substrate. Second, no special equipment beyond that already supplied as standard on most MRI systems is required, providing a (relatively) low

bar to entry from the stand point of equipment. MRS is also a targeted technique, used to investigate the response in a specific region, or regions in the brain, allowing specific hypotheses to be tested, and given the large number of metabolites available for measurement using ^{1}H-MRS a similarly large number of neurochemical hypotheses can be probed.

However, there are some major disadvantages as well. Most metabolites of interest are only present in low concentrations (milli-molar range), and so have a very low signal to noise, such that measurements often need to be averaged over a substantial period of time to gain sufficient signal for reliable fitting and quantification. This leads to long experimental times, or the need for higher field strength, and often both (Lin et al., 2012; Mangia et al., 2007). Spatial resolution is likewise limited by signal to noise, often resulting in the acquisition of signal from a region that contains more brain tissue than just the region of primary activity – which can lead to a dilution in the size of changes seen. This low signal to noise has also lead to researchers using long blocks (> 5mins) of stimulation (Bednařík et al., 2015; Kühn et al., 2015; Lin et al., 2012; Mangia et al., 2008;

Mullins et al., 2005; Schaller et al., 2013) to bolster signal, limiting the temporal resolution of the technique. Appropriate experimental designs can get around some of these limitations however, and with the right designs temporal resolution can be reduced to the order of seconds (Apšvalka et al., 2015; Gussew et al., 2010; Lally et al., 2014), and possibly less.

Neuro-metabolites of interest in fMRS

The first proton fMRS study reported an increase in lactate (~160%) during prolonged visual stimulation (Prichard et al., 1991). This report came out in 1991 – a year after the BOLD effect was first reported and two years before the first report of fMRI using BOLD (Ogawa, Lee, Kay, & Tank, 1990; Ogawa et al., 1993) . The increases in lactate reported in this study were interpreted as reflecting increases in oxidative metabolism during prolonged neuronal stimulation, and so looked to provide an additional window onto brain metabolism during neural activity. Measurements of lactate change have been used to investigate metabolism in response to visual stimuli (Mangia et al., 2007; Peca et al., 2009; Prichard et al., 1991), auditory stimuli (Richards et al., 1997), panic disorders

(Dager et al., 1999) and the effects of exercise on neural activity and metabolism (R. J. Maddock, Casazza, Buonocore, & Tanase, 2011) to name a few. Lactate changes as measured by fMRS have gone through a typical cycle of positive, then negative findings (Boucard et al., 2004), however the latest research firmly supports increased lactate during prolonged neural activity (Bednařík et al., 2015; 2017; Lin et al., 2012; Mangia et al., 2007; 2008; Schaller, Xin, O'Brien, Magill, & Gruetter, 2014) and serves as a good marker for increased metabolic turnover in response to neuronal activity. Investigating lactate changes during neural activity has been useful for the understanding of metabolism – but there are further questions that researchers are interested in, and this is where using fMRS to measure neurotransmitter dynamics in response to neural activity has a role to play.

Of particular interest are the neurotransmitters glutamate, glutamine and GABA. Glutamate (Glu), an amino acid present at around 12 mM (Rae, 2013), is also the major excitatory neurotransmitter within the central nervous system; however its' production by, and involvement in the normal energetic processes of neural cells

can complicate interpretation of changes seen. Given that the relative amount of glutamate involved each process is still not clear, and likely varies with location and compartment (Rae, 2013), interpreting measured changes in glutamate concentration is not always straight forward. Despite this complication, glutamate is of particular interest because as a neurotransmitter it is likely the main component of neural signalling and because alterations in glutamate and glutamatergic signaling are reported to be crucial in several clinical conditions, most notably schizophrenia (Bustillo et al., 2010; Poels et al., 2014; Smesny et al., 2015).

Glutamine (Gln) is synthesized from glutamate in the astrocytes by glutamine synthase, and then shuttled to neurons where it is deaminated to become glutamate again (Rae, 2013; Rothman, Behar, Hyder, & Shulman, 2003). Glutamine is also the major provider of the carbon backbone for GABA (Rae, 2013). It is difficult to separate glutamine from glutamate in standard MRS acquisitions, although appropriate acquisition techniques may allow for reliable detection of

both glutamate and glutamine (Hu et al., 2007; Mullins, Chen, Xu, Caprihan, & Gasparovic, 2008; Wijtenburg & Knight-Scott, 2011).

GABA is synthesized from glutamine by way of glutamate, and is the major inhibitory neurotransmitter in the CNS. Of interest for a number of basic and clinical research questions it is difficult to detect in normal MRS due to low concentrations (small signal) and substantial overlap with peaks from other metabolites (creatine (Cre), glutamate and N-acytel-asparte (NAA)). This has led to the development of several specialized editing sequences to detect GABA reliably, the most commonly applied one being MEGA-PRESS (Mescher, Merkle, Kirsch, Garwood, & Gruetter, 1998; Mullins et al., 2014). Due to the requirement for either a subtraction of at least two acquisitions (MEGA-PRESS), or long acquisition times (2DJ editing methods (Jensen, Frederick, Wang, Brown, & Renshaw, 2005; Jensen et al., 2009; Ryner, Sorenson, & Thomas, 1995)) investigation of GABA dynamics using fMRS has been limited, but there are still several studies worth noting (Cleve, Gussew, & Reichenbach, 2014; Floyer-Lea,

Wylezinska, Kincses, & Matthews, 2006; Michels et al., 2012; Stagg, Best, et al., 2009a).

While the majority of fMRS studies taking place today are focused on these neurotransmitters, or lactate (or both), this does not mean other metabolites are not also being investigated (Castellano, Dias, Foerster, Li, & Covolan, 2012; Lindner, Bell, Iqbal, Mullins, & Christakou, 2017; Sandor et al., 2005).

Meta-analyses of glutamate changes in fMRS

One of the main areas of disagreement in fMRS studies of glutamate dynamics is the effect size, with a wide range of reported glutamatergic changes. For example studies involving pain or noxious stimuli report relatively large increases in glutamate (10-18%) from baseline in the insular cortex and ACC (Cleve et al., 2014; Gussew et al., 2010; Gutzeit et al., 2013; Mullins et al., 2005) while studies of prolonged stimulation report smaller (2-4%) changes in the occipital cortex in response to robust stimulus paradigms like flashing checkerboards (Bednařík et al., 2015; Lin et al., 2012; Mangia et al., 2007). Studies using cognitive task paradigms complicate the matter further as there are reports ranging from a 12

% increases in glutamate upon functional activation in the lateral occipital cortex (Apšvalka et al., 2015) to only 2.6% during a Stroop task in the anterior cingulate cortex (R. Taylor, Schaefer, et al., 2015b). So, the question remains – what is the size of change expected from a fMRS experiment?

To provide an answer to this question, a range of meta-analyses of the existing literature on fMRS measures of glutamate dynamics in varying experimental paradigms were performed. The studies used were drawn from the authors own personal reference library, standing pubmed searches for functional MRS and glutamatergic dynamics and Google scholar searches and recommendations. A pubmed search was also performed on the 26th of May 2017 using the terms "Magnetic resonance spectroscopy", "Functional MRS", "fMRS" and 'functional Magnetic Resonance Spectroscopy" to find additional papers.

Inclusion criteria for this analysis were:

- The article has been written in English and published in a peer-review journal.
- The study utilised proton magnetic resonance spectroscopy as a measurement tool.
- The study investigated Glutamate changes in response to a behavioural or cognitive stimulation (not non-invasive electrical or magnetic stimulation or pharmaceutical interventions).
- The study population consisted of healthy humans, or had a healthy control cohort.
- Metabolite data was acquired in vivo in the human brain using ^{1}H-MRS.

The pubmed searches identified 2560 articles. After careful investigation of titles abstracts, consideration of the inclusion criteria and removal of duplicates between searches, 22 studies were identified for the meta-analyses. The reference

Commented [PM1]: Updated to reflect the inclusion of an additional fMRS study.

lists of the remaining papers were also searched to see if additional papers might be found.

The first basic meta-analysis did not break studies down by region of interest, specific stimulation paradigm, field strength or specific method of fMRS acquisition, instead including all as single data points. In addition, if a study investigated the response in more than one region, more than one stimulation paradigm or paradigm component (eg. Encoding v's retrieval), or more than one type of fMRS acquisition (eg short versus long TE), the response for each region, paradigm, or acquisition, was considered as a separate data point in the meta-analysis (as long as the actual data was separate). This rule allowed all the data from Cleve et al (2014), Maddock et al (2016) and Stanley et al (2017) to be considered. The names of the studies considered for this meta-analysis, the experimental design, and stimulus used are reported in table 1. To allow the meta-analysis to be performed, results from each study were converted to a percent change in glutamate from baseline (or between conditions), the meta-analysis was then performed to find the mean size of glutamate change due to

stimulation, using a maximum likelihood random effects method in the open source meta-analysis software OpenMetaAnalysis (http://www.cebm.brown.edu/openmeta/)(Viechtbauer, 2010; Wallace, Dahabreh, Trikalinos, Lau, & Trow, 2012). The meta-analysis shows that over all studies, a mean change of 6.97 % in glutamate is seen in response to neural activation, with a 95% CI from 5.23 % - 8.70%, and that increase is statistical different from zero at $p < 0.001$. This establishes that it is indeed possible to measure changes in glutamate using proton MRS. However, the forest plot shown in figure 1, demonstrates that several studies report glutamate changes either higher or lower then this range. This is likely a result of several factors, including field strength at which measures were made, experimental design (block versus event related paradigms) and stimulus type – which were not considered in this first analysis.

---------- Figure 1 around here ----------

In general, the size of change detected does differ depending on the magnetic field strength being used, but a recent 7T study also demonstrates an 11% increase of Glutamate on activation (C. Chen et al., 2017), and so the difference in size of change measured between studies done at 4T or lower and those at 7T studies may be a result of something more than just sensitivity of Glutamate detection. It is also important to point out that detection of Glutamate at 3 and 4T has been shown to be reliable with appropriate methodology (Hancu, 2009; Henry, Lauriat, Shanahan, Renshaw, & Jensen, 2010; Hurd et al., 2004; Mullins et al., 2008; Schubert, Gallinat, Seifert, & Rinneberg, 2004; Wijtenburg & Knight-Scott, 2011), so detectability of glutamate may not be the main determinant of differences.

Investigating the experimental paradigm applied, fMRS studies can be split into two main types – Block or event related studies - with long block designs of 4 mins or more prevalent. The use of long blocks versus event related paradigms needs to be carefully considered, as it is possible each are probing different processes in the underlying neurochemical aspects of brain function. In addition

block designs bring with them possible confounds from adaptation, repetition suppression effects (Apšvalka et al., 2015) and homeostatic regulation of signaling. A meta-analysis of all studies involving block related designs shows a mean change of glutamate at 4.749 % (CI's of 3.014% to 5.882%). The forest plot is shown in figure 2. Event related designs however, while fewer in number show a mean change of glutamate as 13.429% (CI's of 9.839% to 17.020%). The forest plot is shown in figure 3.

----------figure 2 and 3 here----------

The results of the two above meta-analysis need to be taken with some caution, as to date, event related studies have only been reported at 3T, while a large number of Block design studies have been acquired at 7T, with a concomitant increase in sensitivity to detect glutamate. It could be argued that this increased sensitivity, makes the data from 7T more reliable, but that does not automatically negate the reliability of 3T data. Block designs have been also performed at 3T, and so a direct comparison between experimental paradigms using data of similar inherent sensitivity can be performed. Doing such a meta-analysis shows that block designs at 3T demonstrate increases in glutamate of 6.66 % (CI's of 4.52 to 8.79),

which while larger than that seen when studies at 7T are considered alone (3.07 % increase, CI's 1.52 to 4.63), is still not as large as the increases reported in event related designs. As it is well known in the neuroimaging world that paradigm design can have a large impact on the results obtained from an fMRI experiment, it would seem prudent to assume that the same may be true for fMRS data, and careful consideration to experimental design should be made before data acquisition begins.

Differences in experimental stimulus also needs to be considered. From table 1 it can be seen that visual stimulation has been the most common stimulation applied to date, with pain next, then other cognitive or physiologic paradigms. A small meta-analysis on visual stimulation shows a mean stimulation effect of 2.318 % (CI of 1.091% to 3.545 %). The forest plot is shown in figure 4. Doing the same for studies using a painful stimulus shows a mean stimulation effect of 14.458 % (CI of 10.722% to 18.193%). The forest plot is shown in figure 5.

----------figure 4 and 5 around here----------

These differences in the size of detected change are important as they impact upon the interpretation of the results from each study. The smaller increase in glutamate seen in visual stimulation studies are generally interpreted as an

increase in oxidative metabolism due to increased neuronal activity. This interpretation is supported by the concomitant increases in lactate, and decreases in aspartate and glucose that are also reported (Bednařík et al., 2015; 2017; Lin et al., 2012; Mangia et al., 2007; Schaller et al., 2013). However, the size of change reported in studies using painful stimuli and event related designs is too high arise from increased metabolic activity alone. Given that these findings have been replicated in several studies from independent labs, they are likely also robust, and so an alternative explanation may need to be sought in these cases. One that is attractive, but which needs to be considered with caution, is that fMRS may be able to index increases in glutamate release in response to stimulation, and that the more salient the stimulus, the greater the response. Similarly, event related designs, which are generally time locked to the stimulus, may have a better temporal specificity for the glutamate changes, and so may index initial glutamate release, while block related designs, which are not necessarily time locked to stimulus onset, may miss these initial glutamate dynamics, and may therefore only be indexing the glutamatergic increases related to energetic processes associated with neural activity (Mangia et al., 2008). There is one other

factor that needs to be considered – a majority of the studies at 7T also utilised short TE MRS, while those at 3T utilised intermediate or long TE. Apart from possible assumptions about the sensitivity and reliability of short versus long TE MRS, there are other factors such as relaxation induced differences in the MRS signal that need to be considered. The implications of these differences will be discussed in the next section.

Where do the changes come from?

While it is tempting to consider that glutamate changes measured with fMRS directly reflect increases in glutamate release into the synapse, this interpretation needs to be approached with caution. MRS is usually not considered selective enough to detect neurochemicals in separate compartments. The glutamate signal detected is typically assumed to come from all cellular compartments within the voxel of interest: the neuronal cytosol; presynaptic vesicles; within the synapse; astrocytic cytosol; and other extra-cellular glutamate pools. If this is the case it is hard to see how Glutamate, or any metabolite, could increase or decrease except through metabolic processes – either anabolism (creation from

precursors) or as a by-product of the catabolism of other metabolites. On this basis, an increase of the order of < 5% is more likely than the average increase of ~ 7 %, and would seem to preclude the average increases of ~ 14% seen in event related studies, especially considering the changes reported there occur within a few seconds. On these grounds, changes larger then 5% are dismissed by some researchers as "impossible".

However, there is a second possible explanation for increases in signal – that of compartmental change, which may explain this discrepancy. During neural activity glutamate released from vesicles may move from one compartment that is less visible to MRS, to one where it becomes more visible, leading to an increase in apparent signal, without an actual increase in "total" concentration. Indeed, work from 1994 proposes that up to 30% of glutamate present within the neuron may not be readily visible to MRS, and that this 30% may be the neurotransmitter pool in presynaptic vesicles (Kauppinen, Pirttilä, Auriola, & Williams, 1994). The mechanism by which the contents of a particular compartment may have reduced MRS visibility is due to a faster T_2 relaxation rate, - the rate at which the MRS

signal decays over time. In simplistic terms, metabolites that are free to tumble and move have longer T_2 relaxation rates, and produce a signal in the MRS experiment for longer after excitation. However, metabolites with restricted movement or tumbling, have faster T_2 relaxation rates, and so the MRS signal from these is only detectable for a short period of time. In fMRS experiments employing short echo times (15 ms or less), these restricted pools (eg. Glutamate in presynaptic vesicles) will contribute more to the total signal in both "rest" and "active" conditions, while in experiments with longer echo times, glutamate in the presynaptic vesicle will have a reduced contribution to the total signal. This means short echo MRS may not be as sensitive to shifts between compartments that may occur during neural activity, reducing the sensitivity to increases in glutamate signal resulting from such a change in compartmentation. In contrast, fMRS experiments with intermediate to long echo times (30 ms or higher) should have a greater sensitivity to such a compartmental shift. As such, larger increases in glutamate in response to neural activity seen in experiments with long echo versus short echo, would support this model of compartmental shift as a mechanism for the observed glutamate level increases.

It is possible to do one final meta-analysis comparing short versus long echo acquisitions. To avoid potential paradigm effects this should also be done only within those studies that have employed block related designs. Doing so produces interesting findings –short echo time experiments (< 15 ms) show an increase in glutamate singal which would be equivalent to a 2.71% increase in concentration (CI's 2.09 to 3.34) while those with intermediate to long echo times (≥ 20 ms) show an increase of 6.42 % (CI's of 4.445 to 8.400). To the authors knowledge only one published fMRS study has investigated the effect of echo time on the size of glutamate changes reported (R. J. Maddock, Casazza, Fernandez, & Maddock, 2016), and they did not report any differences between echo times of 30, 68 or 144 ms. However, as all three echo times are intermediate to long, a lack of a difference does not preclude relaxation effects as a mechanism for the increase in glutamate seen. In addition, as this study was investigating the acute effects of exercise on metabolism (exercise to 80% of Max heart rate, then further exercise under load for a maximum time of 21 minutes), it is not clear if this paradigm would lead to: an increase in neurotransmission within the regions investigated (V1 and ACC); an increase in metabolic turnover;

or both. As such, future fMRS studies utilizing both short and long echo times to investigate glutamate responses to direct neural activity would be helpful to further address, and clarify the compartmental shift hypothesis.

Factors to consider when designing and interpreting fMRS studies

There are a few additional factors that should be considered when devising, and interpreting fMRS studies. One is the possibility of adaptation and more importantly repetition suppression. Just as is seen with fMRI (Grill-Spector & Malach, 2001), the glutamate response may exhibit reductions with repetitive stimuli. Indeed, in an event-related fMRS study, Apsvalka et al (Apšvalka et al., 2015) demonstrated that while novel presentations of line drawings of objects lead to an increase in glutamate measures from baseline, repetition of those same line drawings, no longer elicit an increase in glutamate. This lack of a response for repetitions, was seen whether the data was analyzed looking at blocks containing repeats, or looking at the repeats on their own. Due to the setup of the experimental design, this result also meant that any increased glutamate levels decreased back to baseline within 3 secs, or were driven that

way in response to the repeated stimuli. As such, use of stimuli or paradigms with a high level of repetition may limit detection of glutamate increases. A similar effect has been seen recently in the study of Taylor et al (2015), who investigated the glutamate response in the ACC to two Stroop paradigm blocks, 4 mins in length. While they reported an increase in the glutamate response to the first Stroop paradigm, which returned to baseline during a recovery block, the second Stroop block did not show any increase in healthy controls, and actually showed a decrease from recovery in patients with schizophrenia and major depressive disorder. Similar reductions on repetition where also noticed in a more recent study that combined fMRI and fMRS in an interleaved fashion (Ip et al., 2017). This study involved alternating 64 sec blocks of rest and visual stimulation, and while in general this study reported increases in glutamate within the occipital cortex during visual stimulation as compared to rest, the first rest block (which occurred before any stimulation, and so could be considered a baseline) demonstrated a higher glutamate level than any of the other blocks – rest or stimulation – within the experiment. This decrease in glutamate levels from the first rest block, lends further support to repetitive stimuli having an effect on the

size, and possible direction of change in glutamate seen in fMRS experiments. These are only three studies, but they do highlight that care should be taken when devising fMRS studies to reduce repetition of stimuli if one is trying to maximize the chance of detecting an increase in glutamate.

Similarly, in a typical fMRS experiment, there are several areas in which timing likely plays a key role. The first has already been discussed and refers to elements of timing in the spectroscopic sequence employed in the experiment – particularly the echo time (TE), although the repetition time (TR) should also be considered. As previously discussed, TE in fMRS experiments can range from < 6 ms to 144 ms and usually depends on the specific MRS sequence being employed. While shorter TE leads to more signal in MRS experiment, and hopefully more reliable detection of glutmate, as suggested earlier there are theoretical considerations that would support the use of longer, or at least intermediate, TE to better to ensure detection of any glutamate changes that may occur. However, as comparing the size of change seen between short or long TE

in an fMRS experiment has yet to be rigorously investigated in an empirical study, any suggestions about TE made here, are just that suggestions. For TR, the considerations that usually apply in MRS should be applied to fMRS as well – ensuring enough time between excitations to allow adequate recovery of longitudinal relaxation and hence maximizing the signal received after excitation. As such TR in a fMRS experiment is typically between 2-5 secs, although a TR of 1.5 secs has been utilized successfully (Apšvalka et al., 2015).

MRS experiments are usually collected as multiple repeated acquisitions and averaged on the system to produce one spectrum per set of averages, typically using averages of 128 acquisitions. This may seem to set a lower limit on the temporal resolution possible in fMRS experiments of about 4 mins 16 secs. Collecting the fMRS data as single shot experiments rather than as the standard "average" acquisition can reduce this limit to a resolution of ~ 2 secs, however, single acquisitions have low signal to noise, and so low reliability, and will still require some sort of averaging to produce useful results. There are a few

methods that can be applied here. Some researchers average across participants and within a small number of sequential acquisitions to reach a temporal resolution of 16-20 secs (Bednařík et al., 2015; Ip et al., 2017; Mangia et al., 2007; Schaller et al., 2013). Others have used event related experimental designs to gain an effective temporal resolution at the order of the TR, without compromising on signal to noise. This is done by utilizing trigger pulses from the MRI system at the start of acquisitions to time lock stimulus presentation. Doing so it is possible to "bin" data from specific times within the block (Stanley et al., 2017), or to specific stimulus types (Apšvalka et al., 2015; Lally et al., 2014), or to sparsely space stimuli between "rest" conditions and then bin by stimulus type (Cleve et al., 2014; Gussew et al., 2010). The use of such "event related" design opens up several possibilities and has not been restricted to fMRS studies of glutamate dynamics, having also been applied in studies investigating choline dynamics (Lindner et al., 2017; Nishitani, 2003).

The use of time locking between stimulus delivery and fMRS signal acquisition has another potential benefit and implication – that of mapping the glutamate

response function or "GRF", similar to the mapping of the hemodynamic response function, or HRF, in fMRI. While the full GRF response to stimuli, and the specific timing of changes has not yet been fully described, some aspects have been. Block related designs suggest that within at least 16-20 secs after the start of a stimulation block a small increase in glutamate (3-6%), that likely reflects the increased metabolism associated with neural activity can be seen, and that these increases parallel BOLD signal changes (Ip et al., 2017) measured at the same time. However, these results may only detect one aspect of the glutamatergic response involved with neural activity – the metabolic response of increased energy requirements that accompany activation. Event related studies in contrast, have demonstrated a 9-12% glutamate increase occurring within 300 – 1000 ms after stimulus onset (Apšvalka et al., 2015; Lally et al., 2014), which do not always correlate with the BOLD response. At present, we do not know how this faster response evolves over time, other than that it returns to baseline by 3-4 secs after stimulus onset. Figure 6 presents a hypothetical GRF to a single short "event", the solid line indicating data that has already been collected from two separate studies where the stimulus onset was time locked to occur 300 or 1000

ms before MRS acquisition (Apšvalka et al., 2015; Lally et al., 2014) while the dashed lines represent three of the several possible profiles that may describe the further evolution of this signal. Note this model is speculative, and conceptual only, and studies aimed at collecting further time points to fully describe the GRF by varying the time between stimulus onset and fMRS data acquisition in specific step sizes would be required before any such models are accepted. However, binning across enough different steps, it should be possible to quantify the GRF to a resolution of at least 250 ms, if not better. Performance of these studies at both 3T and 7T would be preferable.

----------Figure 6 around here----------

It is entirely possible that there are two distinct types of glutamate response – one a short, fast and robust response that is related directly to neurotransmitter release, and the other a slower, and smaller response that indirectly reflects increased neurotransmitter release through the increase in metabolism that accompanies it. Much like the early work in fMRI to characterise the HRF, studies

aimed at better identification of the GRF, and elucidating if there are indeed two types of glutamate response, will be extremely useful for both interpretation and design of future neuroscience studies utilising fMRS, and should be considered an essential step in the process of further developing the technique.

The future of fMRS for measuring neurotransmitter dynamics

Looking at table 1, it is apparent that the number of fMRS studies being published each year is increasing – as are the paradigms being utilized. From the meta-analyses, and other discussion it is also clear that the once controversial finding of glutamate increases during neural activity, is now a common finding, but that the size of effect likely depends on several factors including stimulus, experimental design, and timing. As such it may be useful to discuss how to improve the rigor and reliability of future studies.

As with any technique improvements in signal to noise will always be useful. In fMRS studies there are a few main ways in which this can be accomplished. The first is to increase the size of the voxel from which data is acquired. While this will increase the signal to noise of any individual measurement, it creates a partial volume problem where signal is also collected from inactive regions surrounding the site of activity, diluting the size of any signal change that may be detected. As such, this may not be an optimal strategy. Next would be to reduce the TE to decrease the effects of T_2 relaxation on signal – however, as previously discussed this may bias data collected to smaller changes (especially if a compartmental shift of glutamate does indeed have an effect on T2 relaxation). Two other main options remain – increasing the number of averages or number of stimuli per condition in an experiment or increasing field strength. Increasing averages or number of stimuli has a very real experimental time penalty, and can lead to participant fatigue and loss of attention. As such, some method of monitoring performance, and accounting for this in the analysis should be considered – either exclusion of data with poor performance (e.g. remove misses), or some form of weighting by performance. Use of high field systems, if available would

seem to be the easiest way to increase signal, and indeed a majority of fMRS are performed at 7T, however this does not preclude the use of 3T scanners for these studies, as several recent reports have demonstrated that 7T is not essential for successful detection of glutamate dynamics (Apšvalka et al., 2015; Cleve et al., 2014; Gussew et al., 2010; Huang et al., 2015; Kühn et al., 2015; Lally et al., 2014), and reliability of glutamate detection at 3T is likely as good as 7T with appropriate MRS techniques (Mullins et al., 2008; Prinsen, de Graaf, Mason, Pelletier, & Juchem, 2016; Wijtenburg & Knight-Scott, 2011).

In addition to improving signal to noise measures, being more aware of timing issues, particularly the time locking of stimuli and MRS acquisition, are areas that will really improve the rigor and reliability of fMRS studies. Collecting data as a sequence of individual (single TR) dynamic measures, paying attention to how long after stimulus presentation each data point is collected, and binning by different conditions within cognitive paradigms can greatly also increase the informative nature of fMRS studies. The recent paper by Stanley et al (2017) is an

excellent example. Here the researchers applied a working memory paradigm, and thanks to sensible data collection strategies, were able to investigate both encoding and retrieval phases of the paradigm separately, and to even follow the time course of glutamate responses within the task. Taking it a step further, by splitting participants based on performance the authors were able to demonstrate that fast learners had an early rise in glutamate during the encoding phase, while for slow learners the same increase happened much later in this phase, providing more information regarding the role of glutamate in learning then would have been possible if the data had been collected as one measure across the entire cognitive task. Another well know cognitive task, the Stroop test, would similarly benefit from collection of sequential data points and binning across conditions such that possible differences in glutamate responses to congruent versus incongruent conditions could be investigated. Unfortunately the three studies that have investigated the Stroop task (Kühn et al., 2015; R. Taylor, Neufeld, et al., 2015a; R. Taylor, Schaefer, et al., 2015b), while important, have not yet done so, likely because the data were not collected in a fashion that would be conducive to such an analysis. It is hoped that in the future researchers will

give more consideration to these aspects of experimental design and so increase the information gained from experiments of this nature.

Extending fMRS from the study of Glutamate to include GABA and other neurometabolite dynamics is quickly becoming an area of considerable research movement. GABA dynamics particularly are becoming of interest with researchers investigating motor learning (Floyer-Lea et al., 2006), working memory (Michels et al., 2012), pain (Cleve et al., 2014; Kupers, Danielsen, Kehlet, Christensen, & Thomsen, 2009), brain stimulation (Stagg, Best, et al., 2009a; Stagg, WYLEZINSKA, et al., 2009b), and visual stimulation (Mekle et al., 2016). fMRS has also been utilized recently to investigate NAA and NAAG changes in response to visual stimulation (Sandor et al., 2005), and changes in choline in a cognitive paradigm (Lindner et al., 2017). In addition to investigating other metabolites, researchers have shown it is possible to collect fMRS data in conjunction with other measures of neural activity. Both electrophysiology (Lally et al., 2014) and BOLD measures (Apšvalka et al., 2015; Ip et al., 2017) have been collected simultaneously with

fMRS measures of metabolite dynamics. The most recent study of Ip et al (2017) making a major advance in collecting fMRS simultaneously with fMRI measures by interleaving MRS and EPI acquisitions in each TR.

These studies and the others mentioned throughout this review show that MRS is more than just a measure of static neurochemistry, and that investigation of neurotransmitter dynamics is possible – opening up even more windows on the basic functioning of the brain. It is hoped that future researchers will take this technique and apply it to better elucidate the inner workings of the brain and cognition at even finer detail not only in the healthy human brain, but also in conditions where neurotransmitter dysfunction is thought to play a major role (Bustillo et al., 2010; Poels et al., 2013; Schwerk, Alves, Pouwels, & van Amelsvoort, 2013; S. F. Taylor & Tso, 2014).

Table 1 fMRS studies included in the meta-analysis

Study	Date	Region	Stimulus	Exp Design	N
"A Novel Technique to Study the Brain's Response to Pain: Proton Magnetic Resonance Spectroscopy." (Mullins et al., 2005)	2005	ACC	Pain	Block > 5mins	10
"Sustained Neuronal Activation Raises Oxidative Metabolism to a New Steady-State Level: Evidence From 1H NMR Spectroscopy in the Human Visual Cortex." (Mangia et al., 2007)	2007	OCC	Photic Stimulation	Block > 5mins	12
"Insula-Specific Responses Induced by Dental Pain. a Proton Magnetic Resonance Spectroscopy Study." (Gutzeit et al., 2011)	2010	Insula	Pain	Block > 5mins	14
"Time-Resolved Functional 1H MR Spectroscopic Detection of Glutamate Concentration Changes in the Brain During Acute Heat Pain Stimulation.." (Gussew et al., 2010).	2010	Insula	Pain	Event related	6
"Abnormal Changes of Synaptic Excitability in Migraine with Aura." (Siniatchkin et al., 2011)	2011	OCC	Photic Stimulation	Block > 5mins	10
"Vigorous Exercise Increases Brain Lactate and Glx (Glutamate+Glutamine): a Dynamic 1H-MRS Study." (R. J. Maddock et al., 2011)	2011	OCC	Exercise	Intervention	8
"Differential NMR Spectroscopy Reactions of Anterior/Posterior and Right/Left Insular Subdivisions Due to Acute Dental Pain." (Gutzeit et al., 2013)	2012	Insula	Pain	Block > 5mins	
"Investigating the Metabolic Changes Due to Visual Stimulation Using Functional Proton Magnetic Resonance Spectroscopy at 7T" (Lin et al., 2012).	2012	OCC	Photic Stimulation	Block > 5mins	10
"Net Increase of Lactate and Glutamate Concentration in Activated Human Visual Cortex Detected with Magnetic Resonance Spectroscopy at 7 Tesla." (Schaller et al., 2013).	2013	OCC	Photic Stimulation	Block > 5mins	10

"In Vivo Detection of Acute Pain-Induced Changes of GABA+ and Glx in the Human Brain by Using Functional 1H MEGA-PRESS MR Spectroscopy." (Cleve et al., 2014)	2014	ACC	Pain	Event related	15
"Glutamatergic Correlates of Gamma-Band Oscillatory Activity During Cognition: a Concurrent ER-MRS and EEG Study." (Lally et al., 2014)	2014	LOC	Object Recognition	Event related	13
"Are glutamate and lactate increases ubiquitous to physiological activation? A 1H functional MR spectroscopy study during motor activation in human brain at 7Tesla." (Schaller et al., 2014)	2014	Motor cortex	Motor activation	Block 5 mins	11
"Increased Glutamate Levels Observed Upon Functional Activation in the Anterior Cingulate Cortex Using the Stroop Task and Functional Spectroscopy." (R. Taylor, Schaefer, et al., 2015b)	2015	ACC	Stroop	Block 4 mins	7
"Functional magnetic resonance spectroscopy of glutamate in schizophrenia and major depressive disorder: anterior cingulate activity during a color-word Stroop task." (R. Taylor, Neufeld, et al., 2015a)	2015	ACC	Stroop	Block 4 mins	16
"Event-related dynamics of glutamate and BOLD effects measured using functional magnetic resonance spectroscopy (fMRS) at 3T in a repetition suppression paradigm." (Apšvalka et al., 2015)	2015	LOC	Object Recognition	Event related	13
"Neurotransmitter changes during interference task in anterior cingulate cortex: evidence from fMRI-guided functional MRS at 3 T." (Kühn et al., 2015)	2015	ACC	Stroop	Block > 5mins	18
"Increase in glutamate/glutamine concentration in the medial prefrontal cortex during mental imagery: A combined functional mrs and fMRI study." (Huang et al., 2015)	2015	MPFC	Mental Imagery	Block	41
(2015). "Neurochemical and BOLD responses during neuronal activation measured in the human visual cortex at 7 Tesla." (Bednařík et al., 2015)	2015	OCC	Flashing checker	Block > 5mins	12

"Detection of metabolite changes in response to a varying visual stimulation paradigm using short-TE 1H MRS at 7 T." (Mekle et al., 2016)	2016	OCC	board Flashing checker board	Block/Steady state	20
"Acute Modulation of Cortical Glutamate and GABA Content by Physical Activity." (R. J. Maddock et al., 2016)	2016	OCC	Exercise	Intervention	8
"Neurochemical responses to chromatic and achromatic stimuli in the human visual cortex." (Bednařík et al., 2017)	2017	OCC	Coloured checker board		
"Combined fMRI-MRS acquires simultaneous glutamate and BOLD-fMRI signals in the human brain." (Ip et al., 2017)	2017	OCC	Flashing checker board	Block - 1 min	13
"Functional dynamics of hippocampal glutamate during associative learning assessed with in vivo 1H functional magnetic resonance spectroscopy." (Stanley et al., 2017)	2017	Hippocampus	Associative learning - Encoding	Block - 1 min	16
"Activation induced changes in GABA: functional MRS at 7 T with MEGA-sLASER." (Chen et al 2017)	2017	Motor cortex	Hand clenching	Block – 8 min	16

Figure labels

Figure 1: Forrest plot from first meta-analysis of relative Glutamate changes in fMRS studies. Studies are not broken down by region of interest, specific stimulation paradigm, field strength or specific method of fMRS acquisition, instead including all as single data points to give a broad overview of expected changes. In addition, if a study investigated the response in more than one region, more than one stimulation paradigm or paradigm component (eg. Encoding v's retrieval), or more than one type of fMRS acquisition (eg short versus long TE), the response for each region, paradigm, or acquisition, was considered as a separate data point in the meta-analysis (as long as the actual data was separate). The mean size of change in Glutamate is 6.97 % in response to neural activation, with a 95% CI from 5.23 % - 8.72%.

Figure 2: Forrest plot from a meta-analysis of fMRS studies that use a block design paradigm showing a mean relative change of glutamate to stimulation at 4.75% (CI's of 3.3 % to 6.2%).

Figure 3: Forrest plot from a meta-analysis of fMRS studies that use an event related design showing a mean relative change of glutamate to stimulation as 13.429% (CI's of 9.839% to 17.020%).

Figure 4: Forrest plot from a meta-analysis of fMRS studies using visual stimulation shows a mean relative change of Glutamate to stimulation of 2.318 % (CI of 1.091% to 3.545 %).

Figure 5: Forrest plot from a meta-analysis of fMRS studies using a painful stimulus shows a mean relative change of Glutamate to stimulation of 14.458 % (CI of 10.722% to 18.193%).

Figure 6: a) Hypothesised Glutamatergic Response Function (GRF) with three proposed models for the GRF, the first two points are based on initial data sampled at 300 and 1000ms after

stimulus, while the final two points are postulated for three different possibilities, gradual decline to baseline, fast decline to baseline, and rapid decline to below baseline, before a return to baseline. It should be pointed out this is a conceptual model only, and the actual GRF may vary depending on the stimulus type and region investigated. b) Varying the time at which MRS data is collected after stimulus onset in defined increments across multiple events would make it possible to fully map the GRF, with temporal resolution limited by the number of acquisitions required for reliable signal fitting.

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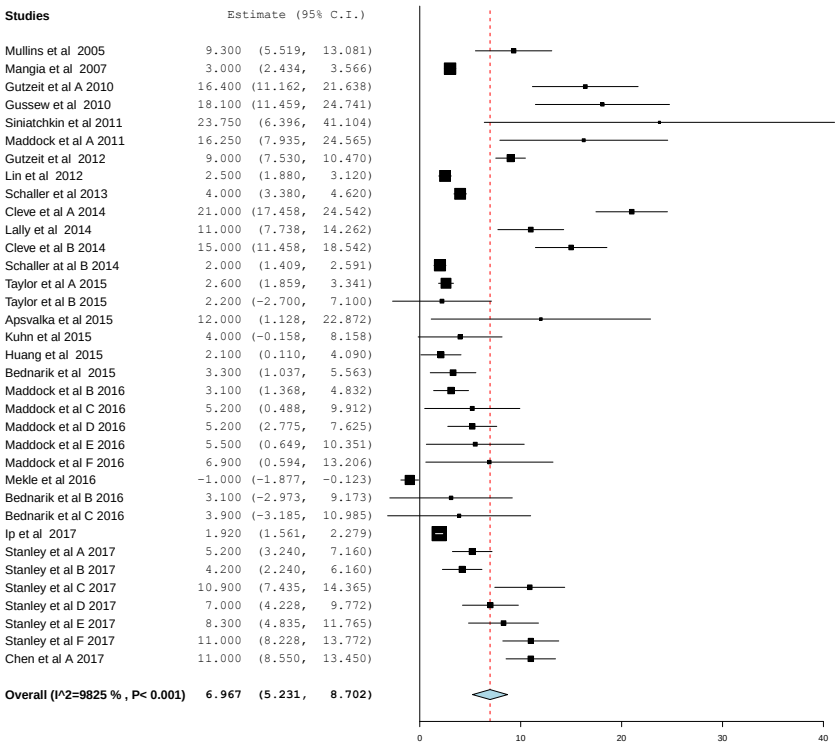

Figure 1: Forrest plot from first meta-analysis of relative Glutamate changes in fMRS studies.

Studies are not broken down by region of interest, specific stimulation paradigm, field strength or specific method of fMRS acquisition, instead including all as single data points to give a broad overview of expected changes. In addition, if a study investigated the response in more than one region, more than one stimulation paradigm or paradigm component (eg. Encoding v's retrieval), or more than one type of fMRS acquisition (eg short versus long TE), the response for each region, paradigm, or acquisition, was considered as a separate data point in

the meta-analysis (as long as the actual data was separate). The mean size of change in Glutamate is 6.97 % in response to neural activation, with a 95% CI from 5.23 % - 8.7%.

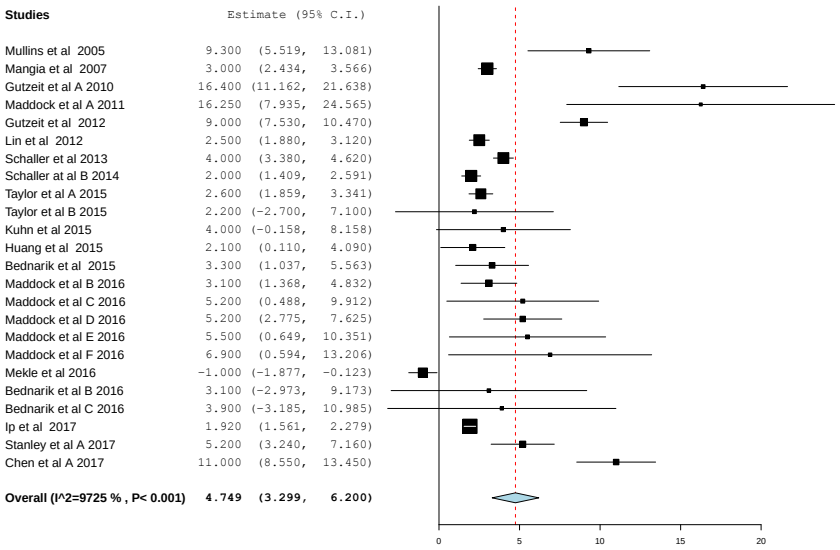

Figure 2: Forrest plot from a meta-analysis of fMRS studies that use a block design paradigm showing a mean relative change of glutamate to stimulation at 4.75% (CI's of 3.3 % to 6.2%).

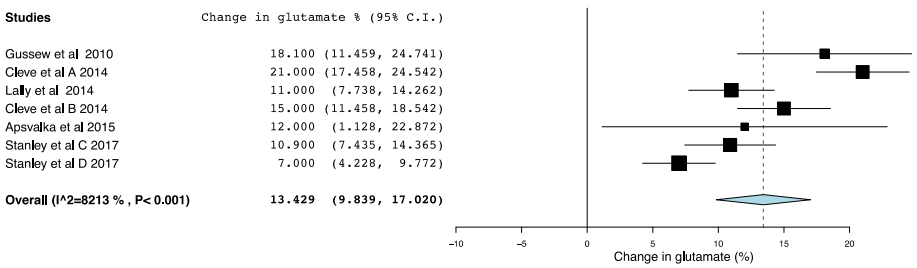

Figure 3: Forrest plot from a meta-analysis of fMRS studies that use an event related design showing a mean relative change of glutamate to stimulation as 13.429% (CI's of 9.839% to 17.020%).

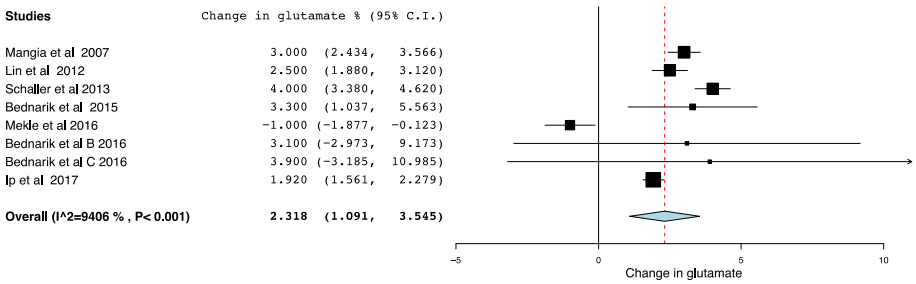

Figure 4: Forrest plot from a meta-analysis of fMRS studies using visual stimulation shows a mean relative change of Glutamate to stimulation of 2.318 % (CI of 1.091% to 3.545 %).

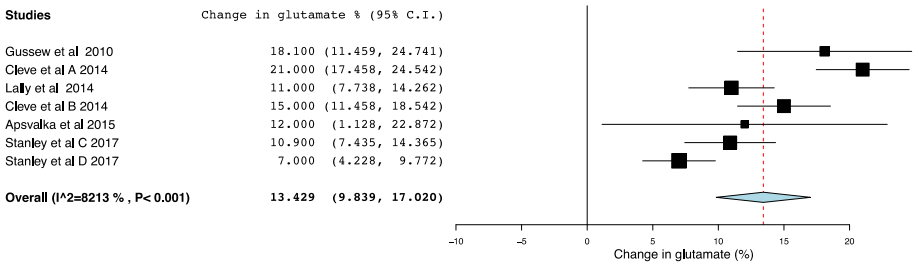

Figure 5: Forrest plot from a meta-analysis of fMRS studies using a painful stimulus shows a mean relative change of Glutamate to stimulation of 14.458 % (CI of 10.722% to 18.193%).

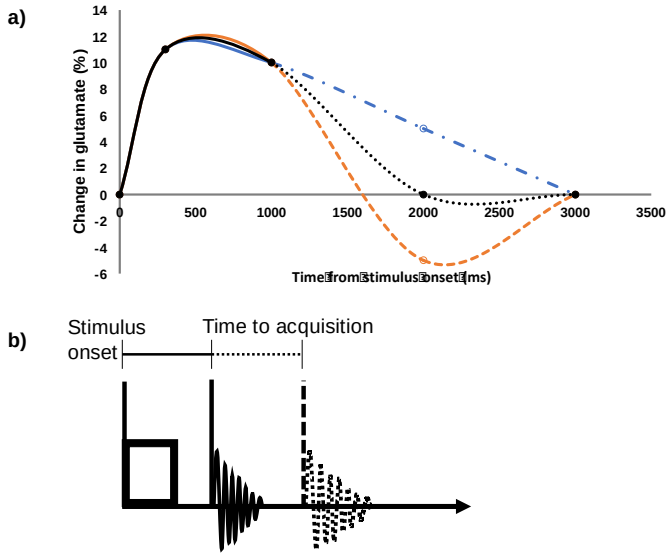

Figure 6: a) Hypothesised Glutamatergic Response Function (GRF) with three proposed models for the GRF, the first two points are based on initial data sampled at 300 and 1000ms after stimulus, while the final two points are postulated for three different possibilities, gradual decline to baseline, fast decline to baseline, and rapid decline to below baseline, before a return to baseline. It should be pointed out this is a conceptual model only, and the actual GRF may vary depending on the stimulus type and region investigated. b) Varying the time at which MRS data is collected after stimulus onset in defined increments across multiple events would make it possible to fully map the GRF, with temporal resolution limited by the number of acquisitions required for reliable signal fitting.